Vol 31, No 10 October 2000

CURRENT CONTENTS

Human PATHOLOGY

- Origin of Giant Cells in Osteoclast-Like Giant Cell Tumors of the Pancreas
- Association of Directors of Anatomic and Surgical Pathology Recommendations
- Rodger C. Haggitt Memorial

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Telomerase in (Pre)neoplastic Cervical Disease

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This study was performed to determine upregulation of the human telomerase RNA component (hTR) and mRNA of the catalytic subunit of telomerase (hTERT) in (pre)malignant cervical lesions, to analyze possible intralesional heterogeneity of hTR expression, and to relate hTR and hTERT mRNA levels to telomerase activity levels and human papillomavirus (HPV) typing. hTR expression was determined by in situ hybridization (ISH) on paraffin-embedded sections, obtained from patients with cervical intraepithelial neoplasia (CIN) I-III or cervical cancer and from normal controls. hTR and hTERT mRNA expression were determined by semiquantitative rt-PCR on frozen samples from the same lesions. Data on telomerase activity and HPV were obtained from a previous study. hTR expression as determined by ISH was observed in 0 of 8 normal cervices, 1 of 14 CIN I, 15 of 28 CIN II, 21 of 30 CIN III, and 16 of 18 cervical cancer specimens. In general, hybridization patterns for hTR expression were homogeneous throughout the lesion. Frequency of hTR expression was related to grade of CIN/cervical cancer ($P < .001$). hTR expression, as determined by rt-PCR, was detected in 8 of 8 normal cervices, 2 of 2 CIN I, 12 of 14 CIN II, 23 of 23 CIN III, and 16 of 17 cervical cancer specimens. hTERT mRNA was detected in 1 of 8 normal cervices, 1 of 2 CIN I, 5 of 14 CIN II, 14 of 23 CIN III, and 11 of 17 cervical cancer specimens. hTR as determined by rt-PCR was not related to grade of CIN/cervical cancer, whereas hTERT mRNA expression was related to grade of CIN/cervical cancer ($P < .01$). hTR expression, as determined by ISH and hTERT mRNA expression by rt-PCR, were related to telomerase activity levels ($P < .001$, $P < .05$, respectively) and presence of oncogenic types of HPV (both $P < .05$). Our data show frequent upregulation of hTR and hTERT mRNA expression in CIN lesions, which appear to occur earlier than induction of telomerase activity. The fact that semiquantitative hTERT mRNA as well as hTR levels are related to telomerase activity levels illustrates that in (pre)malignant cervical lesions upregulation of both telomerase components may be important for functional telomerase. HUM PATHOL 31:1304-1312. Copyright © 2000 by W.B. Saunders Company

Key words: telomerase, hTR, hTERT, (pre)malignant cervix.

Abbreviations: hTR, human telomerase RNA component; hTERT, human telomerase reverse transcriptase; rt-PCR, reverse transcription polymerase chain reaction; HPV, human papillomavirus; CIN, cervical intraepithelial neoplasia; ISH, in situ hybridization; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetra-acetic acid; DTT, dithiothreitol; M-ITAS, modified internal telomerase assay standard; GAPDH, glyceraldehyde-3-phosphatase dehydrogenase.

Telomerase is a ribonucleoprotein enzyme that replaces short stretches of repeat nucleotides (TTAGGG) that are lost from telomeric ends of chromosomes with each round of replication. The RNA component of telomerase contains a segment that is used as template.[1] The human telomerase RNA component (hTR) has been cloned by Feng et al.[2] Recently, protein components of the human telomerase also have been identified, the telomerase-associated protein TP1[3] and the catalytic subunit hTERT.[4-6] At present, hTERT is often considered to be the rate-limiting component in the formation of functional telomerase.[7-10] However, presence of hTR also appears to be necessary for functional telomerase.[11,12] In proliferating normal somatic cells, absence of telomerase activity results in progressive telomere shortening to a critical level, at which point normal cells will senesce.[13] Studies both in cancer cell lines as well as in human malignant tumor specimens, using sensitive polymerase chain reaction (PCR)-based telomerase activity assays, have shown that, in contrast to normal somatic cells, the large majority of malignant cells (>95%) are characterized by telomerase activity and thereby unrestricted proliferative capacity.[14,15] Because of its unique expression in cancer cells compared with (most) normal somatic cells, determination of telomerase activity has been suggested as a biomarker for the early detection of cancer.

Ex vivo, in human papillomavirus (HPV) transformed cervical cells low levels of telomerase activity were already found before immortalization.[15] Increased telomerase activity has also been reported in the large majority (>90%) of cervical cancer specimens, whereas data on telomerase activity in premalignant cervical lesions or cervical intraepithelial neoplasia (CIN) are not unambiguous.[16-23] In a previous study, we reported that in frozen sections presence and levels of telomerase activity were related to grade of CIN/cancer. However, we also observed that telomerase activity levels as determined in cervical scrapings were only weakly related to telomerase activity levels in frozen sections from the same cervical lesion.[21] Among other reasons, this frequent discrepancy may be attributable to heter-

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ogeneity of telomerase activity in (pre)malignant cervical lesions. Studies using in situ hybridisation (ISH) techniques for the expression of hTR have not only shown dysregulation of telomerase activity and hTR expression, but also important heterogeneity in hTR expression, especially in premalignant lesions of different tumour types.[24-26]

The aims of the current study were (1) to determine upregulation of hTR expression in (pre)malignant cervical lesions by ISH and reverse transcriptase PCR (rt-PCR), 2) to determine hTERT mRNA expression by rt-PCR, and (3) to relate hTR and hTERT mRNA expression to telomerase activity levels and HPV typing. In addition, application of an ISH technique allowed the assessment of possible heterogeneity of hTR expression within the cervical lesions.

PATIENTS AND METHODS

Tissue Samples

Tissue samples were obtained from patients who participated in a previously reported study.[21] In short, these patients were referred to our outpatient clinic for an abnormal cervical smear. Treatment policy was as follows: patients referred for 2 smears with mild or 1 smear with moderate dysplasia had colposcopically directed biopsies. In case of CIN I, cytologic follow-up was performed. In case of CIN II or III, the transformation zone was excised 4 to 6 weeks later by loop excision. Patients referred for severe dysplasia or carcinoma in situ in 1 smear underwent colposcopy and loop excision of the transformation zone at the same visit. As normal controls, specimens of the cervix were obtained from patients planned for hysterectomy for noncervical nonmalignant disease. As cervical cancer controls, specimens were obtained from patients already diagnosed with squamous cell cancer of the cervix and planned for radical hysterectomy. Patients with cervical cancer were staged according to FIGO guidelines.[27] The study was approved by the medical ethics committee of our hospital. Informed consent was obtained from all patients.

Pathologic Examination

CIN was diagnosed and graded according to the criteria of the World Health Organisation.[28] The cervical neoplasia was classified according to the most severe lesion found by histologic examination. All cervical cancers were classified into well (grade 1), moderately (grade 2), poorly (3), or undifferentiated (grade 4) squamous cell carcinoma.[29] All examinations were performed by 1 experienced gyn-pathologist (H.H.).

Frozen Specimens and Cell Lines

Fresh specimens from patients undergoing (radical) hysterectomies or loop excision of the transformation zone were obtained as described by Wisman et al.[21] Ten consecutive frozen sections (25 μm) were cut, and the first and last section were stained with hematoxylin and eosin. The remaining 8 sections were available for the TRAP assay and for RNA isolation. Two sections per TRAP assay were lyzed in 100 μL TRAP lysis buffer (0.5% CHAPS; 10 mmol/L Tris/HCl (pH 7.5); 1 mmol/L $MgCl_2$; 1 mmol/L ethyleneglycoltetra-acetic acid; 10% glycerol; 5 mmol/L β-mercaptoethanol; 0.1 mmol/L phenylmethyl sulfonyl fluoride and incubated on ice for 25 min. After centrifugation at 15,000*g* for 20 minutes at 4°C, the supernatant was quickly frozen in liquid nitrogen and stored at −80°C until further processing. Four frozen sections were used for the isolation of high-quality RNA.

GLC_4, a human small cell lung cancer cell line, derived from a pleural effusion,[30] was used for standardisation of the semi-quantitative TRAP assay. This cell line is kept in continuous culture in RPMI 1640 medium (Life Technologies, Breda, the Netherlands) supplemented with 10% fetal calf serum (Life Technologies, Breda, the Netherlands). For TRAP assay, cells were isolated according to Wisman et al.[21]

In Situ Hybridization

Preparation and Pretreatment of Tissue Sections. Formalin-fixed paraffin-embedded blocks were cut in sections of 5 μm. Tissue sections were deparaffinized (2 times 10 minutes with histoclear), rehydrated (100%, 90%, 70%, 50% and 30% EtOH, 10 seconds each), rinsed for 5 minutes in 0.85% NaCl, followed by 5 minutes in phosphate-buffered saline (PBS: 6.4 mmol/L Na_2HPO_4; 1.5 mmol/L KH_2PO_4; 0.14 mmol/L NaCl; 2.7 mmol/L KCl [pH = 7.2]). Sections were fixed in 4% paraformaldehyde (in PBS) for 20 minutes, washed 5 minutes in PBS, and treated in proteinase K buffer (40 mg/mL proteinase K in 50 mmol/L Tris/HCl [pH = 7.5]; 5 mmol/L ethylenediaminetetra-acetic acid [EDTA]) for 7.5 minutes. Slides were washed in PBS 2 minutes and post fixed in 4% paraformaldehyde for 5 minutes. Sections were washed in H_2O and acetylated in freshly prepared 0.25% acetic anhydride, 0.1 mmol/L triethanolamine for 10 minutes. Sections were washed 5 minutes in 0.85% NaCl, 5 minutes PBS, and were dehydrated (30%, 50%, 70%, 90%, and 100% EtOH, 10 seconds each) before hybridization.

Probe Preparation. The riboprobe plasmid containing telomerase RNA sequences used for RNA ISH is as previously described by Soder et al.[31] Probes were labeled with (^{35}S)-UTP by using an RNA labeling kit (Amersham, Buckinghamshire, UK). Transcripts were purified with a Sephadex G-50 column (Pharmacia Biotech. Benelux, Roosendaal, the Netherlands), phenol/chloroform extracted, precipitated in ethanol, and resuspended in 50 mmol/L dithiothreitol (DTT).

Hybridization and Washing Procedures. Hybridization and washing procedures were performed according to Soder et al.[26] Briefly, sections were hybridized overnight in hybridization buffer (60%, formamide; 10% dextran sulphate; 2× SSC; 10 mmol/L Tris/HCl [pH = 7.5]; 1× Denhardts; 0.1% sodium dodecyl sulfate; 0.4 mg/mL tRNA; 0.2 mg/mL salmon DNA; 50 mmol/L DTT; 50,000 cpm/mL probe) at 52°C. Sections were washed 30 minutes in 5× SSC, 0.1% β-mercaptoethanol at 50°C, 20 minutes in 50% formamide, 2× SSC, 1% β-mercaptoethanol at 65°C, and twice 10 minutes in RNase A buffer (0.5 mmol/L NaCl; 10 mmol/L Tris/HCl [pH = 7.5]; 5 mmol/L EDTA) at 37°C. Sections were treated with 20 mg/mL RNase A in RNase buffer at 37°C for 30 minutes, to degrade all single-stranded unhybridized probe. Sections were then washed 15 minutes in RNase buffer at 37°C, 20 minutes in 50% formamide, 2× SSC, 1% β-mercaptoethanol at 65°C, 15 minutes in 2× SSC at 50°C, 15 minutes in 0.1× SSC, and dehydrated. Slides were coated with 0.1% gelatine, 0.01% chrome alun. After drying, slides were dipped in light microscopy emulsion (Amersham, Buckinghamshire, United Kingdom) and exposed for 10 days in light-tight boxes at 4°C. Slides were developed in 20% phenisol for 2.5 minutes, washed in 1% acetic acid and H_2O each for 30 seconds, fixed in 30% sodium thiosulfate for 5 minutes, rinsed in tap water for 30 minutes, and counterstained with hematoxylin.

The sense probe was used for hybridization as a negative control, and a non–small cell lung cancer specimen was used as a positive control, as described by Soder et al,[31] which allows specific hTR signals. hTR expression in individual sections was scored as follows: −, no detection; +, moderate hTR expression, and ++, hTR expression was comparable to the positive control, which was high. All experiments were performed in duplicate. After scoring, results were divided into 4 categories: negative, −/−; weak, +/−; moderate, +/+; and high ++/++. All sections were read independently by an investigator (G.B.A.W.) and a pathologist (H.H.).

TRAP Assay

The TRAP assay was performed as previously described by Wisman et al.[21] In short, telomerase activity levels in the cervical specimen were determined with a fluorescence-based telomeric repeat amplification protocol assay by using GLC_4 cells as standard in each assay. Peaks representing telomerase activity in GLC_4 cell equivalents were summed, then relatively expressed to telomerase activity of 100 GLC_4 cell equivalents (set at 100%) and normalized to the signal of the modified internal telomerase assay standard (M-ITAS). For the samples (1 μg and 0.1 μg), the peaks representing telomerase activity were also summed and normalized to the signal of M-ITAS; thereafter, the relative telomerase activity of the specimen was correlated to GLC_4 cell number (relative quantification comparable to 10 GLC_4 cell equivalents = 10 U/μg protein). Data on telomerase activity were as reported before.[21]

Reverse Transcriptase PCR

Isolation of RNA and Reverse Transcriptase PCR High-quality RNA was isolated by lyzing 4 sections of 25 μm in 500 μL guanidine thiocyanate buffer (4 mmol/L guanidine thiocyanate; 0.5% n-lauroyl sarcosine; 25 mmol/L sodium citrate [pH 7.0], 0.1 mol/L β-mercaptoethanol) according to Meersma et al.[32] The pellet was solubilized in 25 μL DEPC-H_2O. Before cDNA synthesis, RNA was treated with DNase (Roche Diagnostics, Almere, The Netherlands). cDNA was synthesized from 2 μL cervical RNA as described by the manufacturer's protocol (Life Technologies, Breda, the Netherlands) by using $T_{11}VN$ oligo's using 1 μg of GLC_4 total RNA cells as standard in each assay. Although hTR is not polyadenylated, it is possible to synthesize cDNA of hTR by using the $T_{11}VN$ oligos because of the sequence: ACA_6 from base 169 to base 176[2]. Rt-PCR was performed separately for hTERT mRNA, hTR, and a housekeeping mRNA, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) by using the following primers: 5′-CGC AAG ACT GTC TGG AGC AA-3′ (forward) and 5′-GGA TGA AGC CGA GTC TGG A-3′ (reverse) for amplifying hTERT mRNA[33] (35 cycles), 5′-TCT AAC CCT AAC TGA GAA GGG CGT AG-3′ (forward) and 5′-GTT TGC TCT AGA ATG AAC GGT GGA AC-′3 (reverse) for amplifying hTR (35 cycles), and 5′-CAC CAC CAT GGA GAA GGC TGG-3′ (forward) and 5′-CCA AAG TTG TCA TGG ATG ACC-3′ (reverse) for amplifying GAPDH[33] (30 cycles). Subsequent dilutions of cDNA were used for rt-PCR. DNA products were electrophoresed in a 2% agarose gel in 1× Tris-borate EDTA buffer, resulting in DNA fragments of 145 bp, 280 bp, and 200 bp, respectively for hTR, hTERT, and GAPDH. DNA was denatured in 0.5 mol/L NaOH and 1.5 mol/L NaCl and neutralized in 0.5 mol/L Tris/HCl (pH = 7.5) and 1.5 mmol/NaCl, after which the DNA was transferred to a positively charged nylon membrane (Roche Diagnostics, Almere, The Netherlands) using 10× SSC ON and dried for 2 hours at 80°C. Prehybridization, hybridization with probe, preincubation, incubation with 1:5,000 alkaline phosphatase–conjugated streptavidine, and washing were performed according to biotin luminescence detection kit instructions (Roche Diagnostics, Almere, the Netherlands). DNA products were visualized by incubation of the membrane with the chemiluminescence substrate CPD-Star (1:100), according to the supplied instructions (Tropix, Westburg, Leusden, the Netherlands) and exposure to a film.

Films were scanned, and densitometry analysis was performed with Diversity One PDI software (Pharmacia Biotech, Roosendaal, the Netherlands). GAPDH, hTR, and hTERT levels in GLC_4 were set at 100%. Expression levels in the samples were relatively expressed to the expression levels in GLC_4, whereafter the expression levels were normalized to the housekeeping gene GAPDH. hTR and hTERT expression were scored as follows: negative, no detection; very low, expression <10% GLC_4; low, 10% ≤ expression < 75% GLC_4; moderate, 75% ≤ expression < 200% GLC_4; high, 200% ≤ expression < 1000% GLC_4; very high, 1,000% ≤ expression < 2000% GLC_4. The linearity of the densitometry was 0.98, when the signal is not saturied and not too faint. To accomplish a correct measurement of the signal, several dilutions were made of the cDNA. All experiments were performed in duplicate.

Probe Preparation. The probes used for rt-PCR were labeled with 0.4 mmol/L biotin-14-ATP by using PCR with the DNA products of the rt-PCR as template (Roche, Almere, The Netherlands). Transcripts were purified with a PCR column (Qiagen, Westburg, Leusden, the Netherlands).

HPV Typing

DNA was prepared from pellets obtained from cervical scrapings by using the guanidium isothiocyanate-diatom procedure.[34] HPV typing was performed as described previously.[21]

Statistics

Associations between numerical parameters were analyzed with the chi-square test with Yates' correction for small numbers. Rank correlations were calculated by the method of Spearman. All tests were 2-sided; only *P*-values < .05 were considered significant.

RESULTS

Patients

From December 1995 to April 1997, 77 consecutive patients with abnormal cervical smears were included in the study. Final histologic diagnoses were CIN I, $n = 14$; CIN II, $n = 28$; CIN III, $n = 30$, and cervical cancers: $n = 5$ (FIGO stage Ia: $n = 1$, Ib: $n = 3$, IIa: $n = 1$). Paraffin-embedded tissue was available from all of these patients (from either biopsies or loop excision samples). As controls, 8 normal cervix and 13 cervical cancer specimens with FIGO stage Ib ($n = 4$), IIa ($n = 7$), IIb ($n = 1$), and IV ($n = 1$) squamous cell cervical cancer were obtained from patients consecutively treated in our hospital in the same period. Frozen samples for telomerase activity were available from 74 of 98 patients, because frozen sections from CIN or cancer patients were only used in the study when in the first and last hematoxylin and eosin–stained sections dysplastic/tumor cells were observed. Because of the small sample and the fact that samples were only eligible when dysplastic cells were present in the first and

last section, frozen samples for RNA isolation were available from 64 patients. Because our treatment policy in patients with CIN I routinely is cytologic follow-up, frozen sections were only obtained from 2 patients with CIN I in their biopsy who opted themselves for excision of the lesion. All biopsy specimens from patients with CIN contained 1% to 10% of dysplastic cells.

hTR In Situ Hybridization

Figure 1 shows representative examples of in situ hTR expression pattern. Figure 1A shows no hTR expression, whereas Figure 1B and 1C shows moderate hTR expression in a CIN III lesion and a cervical cancer lesion. The normal surrounding cells again did not exhibit hTR expression. Figure 2A and Table 1 summarize hTR expression determined by ISH in the different categories of cervical lesions. hTR expression was not observed in normal cervices ($n = 8$). In 1 of 14 (7%) CIN I lesions, hTR was moderately expressed. In CIN II lesions, 15 of 28 (54%) showed hTR expression. Of these 15 positive lesions, 10 (36%) showed weak, 4 (14%) moderate, and 1 (4%) high hTR expression. In 21 of 30 (70%) CIN III lesions, hTR expression was found. From these 21 positive lesions, 5 (17%) showed weak, 12 (40%) moderate, and 4 (13%) high hTR expression. In cervical cancers, 16 of 18 (89%) were hTR positive. From these 17 positive specimens, 3 (17%) were weak, 10 (55%) moderate, and 3 (17%) high for hTR expression. Levels and presence of hTR expression were related to grade of CIN and cervical cancer ($r = 0.59$, $P < .001$; $r = 0.56$, $P < .001$, respectively). Although semiquantitatively determined, moderate or high hTR expression was more frequently observed in CIN III/cervical cancer lesions in comparison with CIN II lesions ($P < .001$), pointing to higher hTR expression levels in higher-grade lesions.

In general, hybridization patterns for hTR expression were homogeneous throughout the hTR-positive CIN and cervical cancer lesions, and no important intralesional heterogeneity was observed. In lower-grade lesions, the hTR-positive cells were primarily observed in the (dysplastic) cells of the basal layer, whereas in higher-grade lesions, hTR-positive cells were observed throughout the full thickness of the lesion.

hTR and hTERT mRNA Reverse Transcriptase PCR

Figure 3 shows a representative hTR and hTERT mRNA rt-PCR in 2 cervical specimens. Figure 2B, Figure 2C, and Table 1 summarize the levels and positivity of hTR and hTERT mRNA in CIN lesions and cervical cancer specimens as determined by rt-PCR. In contrast to hTR as determined by ISH, hTR expression as determined by rt-PCR was detected in almost every normal cervix, CIN, and cervical cancer lesion. Although the presence of hTR by rt-PCR was not related to grade of CIN/cervical cancer, semiquantitative levels of hTR expression showed a trend with grade of CIN/cervical cancer ($r = 0.24$, $P = .06$). Semiquantitative hTR expression levels as determined by rt-PCR showed a trend with hTR levels as determined by ISH ($r = 0.23$, $P = .07$). hTERT mRNA was expressed in 1 of 8 (13%) normal cervices, in 1 of 2 (50%) CIN I, in 5 of 14 (36%) CIN II, in 14 of 23 (61%) CIN III, and in 11 of 17 (65%) cervical cancer lesions. Semiquantitative levels and presence of hTERT mRNA were related to grade of CIN/cervical cancer ($r = 0.32$, $P < .01$, both).

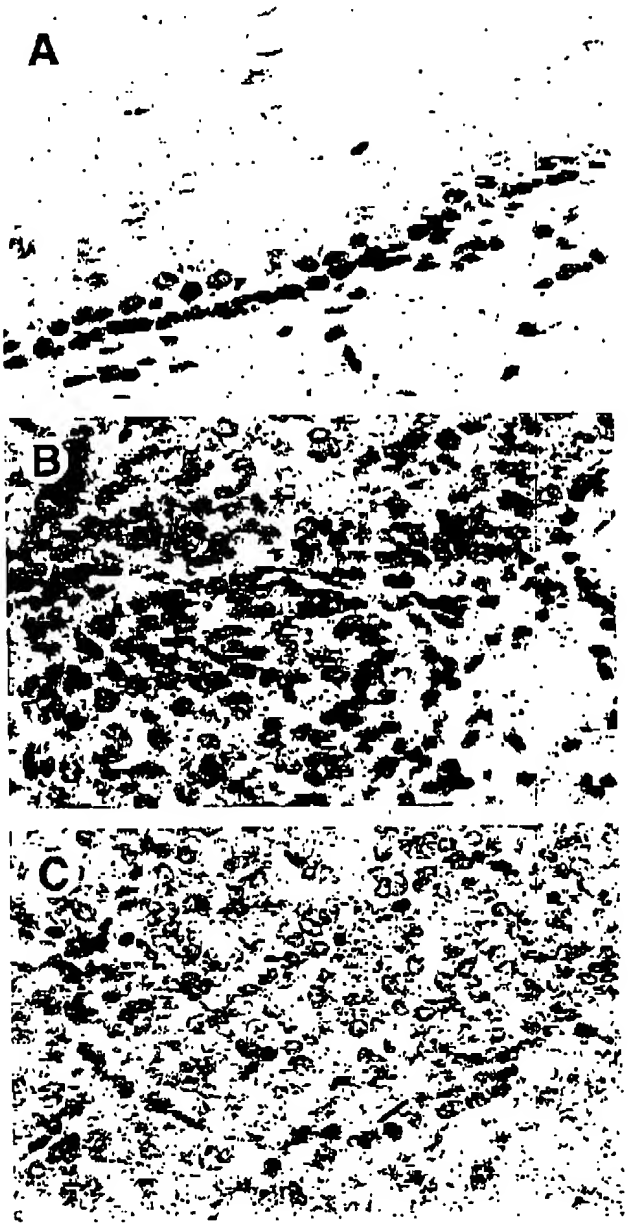

FIGURE 1. In situ detection of hTR expression. (A) No hTR expression in a normal cervix. (Original magnification ×400.) (B) Moderate hTR in a cervical intraepithelial neoplasia (CIN) grade III lesion. (Original magnification ×400). Normal surrounding cells did not exhibit hTR expression. (C) Moderate hTR in a cervical cancer lesion. (Original magnification ×400.)

HUMAN PATHOLOGY Volume 31, No. 10 (October 2000)

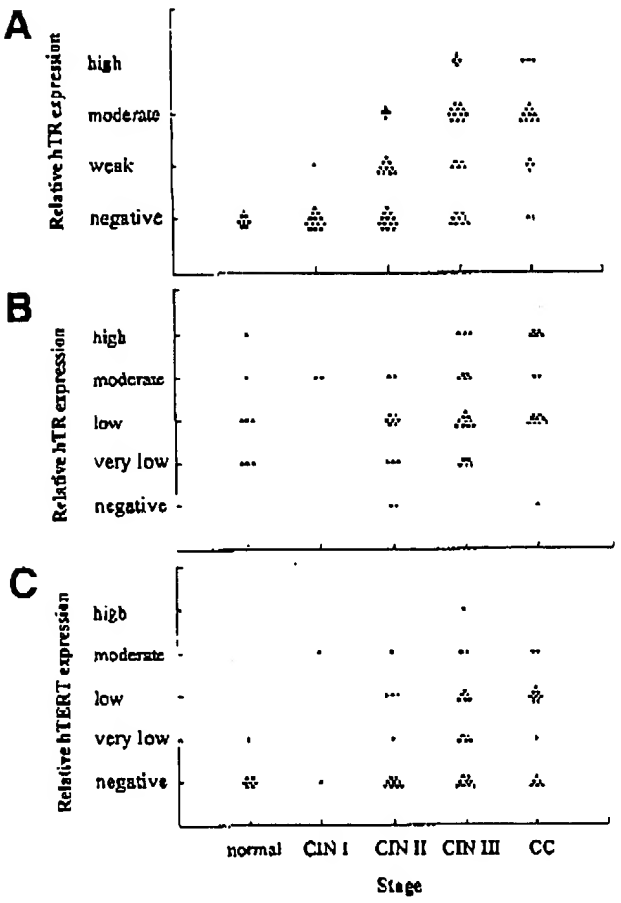

FIGURE 2. Relative hTR expression as determined by ISH in paraffin-embedded material (A), relative hTR expression (B), and hTERT mRNA (C), as determined by rt-PCR in normal cervices, CIN I-III, and cervical cancer (CC).

hTR and hTERT mRNA Expression in Relation to Telomerase Activity

Data on telomerase activity were obtained from a previous report.[21] Figure 4 shows the relation between hTR expression levels as determined by ISH and telomerase activity levels, whereas Table 2 shows presence of hTR in relation to presence of telomerase activity. Five

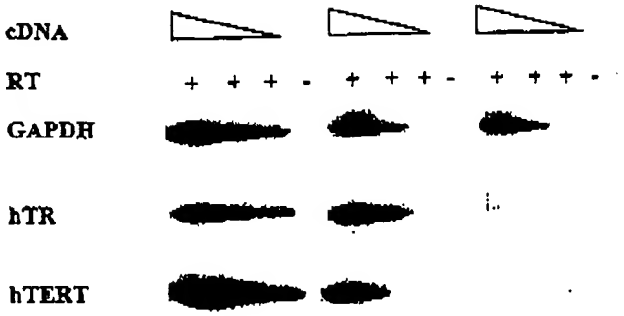

FIGURE 3. Representative blot of GAPDH, hTR, and hTERT rt-PCR in a cervical cancer specimen, a CIN III lesion, and a normal cervical specimen. Subsequent dilutions (0*, 5*, and 25*) of cDNA were used for rt-PCR.

of 29 (17%) specimens negative for hTR showed telomerase activity, of which 1 sample showed very high telomerase activity. Telomerase activity was observed in 4 of 13 (31%) specimens with weak, 14 of 24 (58%) specimens with moderate, and 6 of 8 (75%) specimens with high hTR expression. hTR expression levels and presence by ISH were related to telomerase activity levels and presence ($r = 0.43$; $P < .001$; $r = 0.36$, $P < .01$, respectively). In 21 lesions hTR expression (9 weak, 10 moderate, and 2 high) was observed, whereas no telomerase activity was detected.

Figure 5 and Table 2 show hTR and hTERT mRNA expression levels and presence as determined by rt-PCR in relation to telomerase activity. Semiquantitative hTR levels as well as hTR presence were not related to semiquantitative levels or presence of telomerase activity. Presence of hTERT mRNA expression was related to positivity of telomerase activity ($r = 0.29$, $P < .05$), whereas semiquantitative levels of hTERT mRNA showed a trend with telomerase activity levels ($r = 0.23$, $P = .07$). However, 23 specimens were discordant for hTERT mRNA expression and telomerase activity.

hTR and hTERT mRNA Expression in Relation to HPV Type

HPV data were available for 85 of 98 patients.[21] Table 3 shows hTR expression as determined with ISH in relation to HPV type in the different categories of

Table 1. Prevalence of Telomerase Components in Normal Cervical Specimens, CIN I-III, and Cervical Cancer (CC) Specimens

	Normal (%)	CIN I (%)	CIN II (%)	CIN III (%)	CC (%)
ISH					
hTR	0/8 (0%)*	1/14 (7%)*	15/28 (54%)	21/30 (70%)*	16/18 (89%)*
rt-PCR					
hTR	8/8 (100%)	2/2 (100%)	12/14 (86%)	23/23 (100%)	16/17 (94%)
hTERT mRNA	1/8 (13%)†	1/2 (50%)†	5/14 (36%)†	14/23 (61%)†	11/17 (65%)†

Note. r and P-value were presented if $P < .05$: relation between telomerase components and grade of CIN/cervical cancer.

Abbreviations: ISH, in situ hybridization; rt-PCR, reverse transcription polymerase chain reaction; hTR, human telomerase RNA component; hTERT, human telomerase reverse transcriptase.

*$r = 0.56$; $P < .001$.

†$r = 0.32$; $P < .01$.

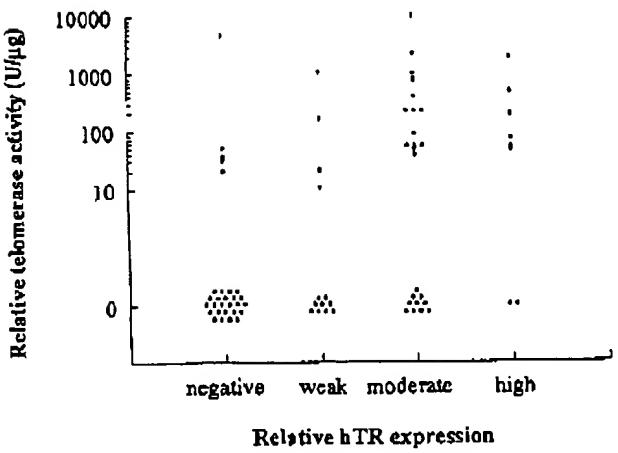

FIGURE 4. Relative hTR expression as determined by ISH in relation to telomerase activity levels as determined in the same lesions.

lesions. Oncogenic HPV was detected in 76 of 86 patients (90%) (HPV 16: $n = 60$ [71%], other oncogenic HPV types [18, 31, 33, 52, 56, 58, 66, 68, 73]: $n = 16$ [19%]). HPV 6 was detected in 1 patient, whereas in 8 patients (9%) no HPV DNA was detected. hTR expression levels and presence of hTR were related to presence of oncogenic HPV ($r = 0.27$, $P < .05$; $r = 0.24$, $P < .05$, respectively). No hTR expression was found in 6 of 8 HPV-negative lesions (no CIN: $n = 1$, CIN II: $n = 4$, CIN III: $n = 1$), whereas detectable hTR expression was observed in 49 of 76 oncogenic HPV-positive lesions.

HPV data were available from 52 of 64 patients analysed for hTR and hTERT mRNA expression, as determined by rt-PCR (data not shown). Detectable hTR expression was observed in all 5 HPV-negative lesions and in 44 of 46 oncogenic HPV-positive lesions. Semiquantitative hTR expression levels and presence of hTR as determined by rt-PCR were not related to presence of oncogenic HPV. hTERT mRNA expression was not detected in 5 of 6 HPV-negative lesions, whereas hTERT mRNA was observed in 28 of 46 oncogenic HPV-positive lesions. Presence of hTERT mRNA was related to presence of oncogenic HPV ($r = 0.28$, $P < .05$).

DISCUSSION

Our study shows that higher semiquantitative expression of both hTR, as detected by ISH, and hTERT mRNA, as determined by rt-PCR, are related to higher grade of (pre)malignant cervical lesions. In a previous study, we already reported that upregulation of telomerase activity also occurs more frequently in higher-grade CIN and cervical cancer lesions.[21] Our combined data suggest that hTR and hTERT mRNA are frequently upregulated in an earlier stage of cervical carcinogenesis than telomerase activity, because, in contrast to telomerase activity, higher levels of hTR and hTERT mRNA were already detected in most CIN II lesions. By rt-PCR, a much more sensitive assay than ISH, hTR was detected in virtually all lesions, thereby precluding any further analysis of relations between presence of hTR by rt-PCR with other parameters, such as telomerase activity, HPV type, etc. However, because our rt-PCR has a semiquantitative character, we were able to relate semiquantitative hTR levels to other parameters. The fact, however, that we only observed a trend between hTR levels as determined by ISH and rt-PCR illustrates the true semiquantitative character of both assays.

Recently, Yashima et al[25] also examined hTR expression in (pre)malignant cervical lesions by ISH. In their study, weak hTR expression was present in normal and low-grade CIN specimens ($n = 21$), mostly limited to the basal layer, whereas all high-grade CIN ($n = 14$) and cervical cancers ($n = 13$) displayed hTR expression. In concordance with our study, upregulation of hTR expression was related with severity of the underlying lesion. Soder et al[26] reported hTR expression, as determined by ISH, in 29 of 67 (43%) cervical cancer lesions and in 8 of 20 (40%) premalignant cervical specimens. In contrast to our study, no data were available in these 2 studies on telomerase activity levels in the specimens analyzed for hTR expression.[25,26]

In our study, hTR expression by ISH was related to telomerase activity levels, although some specimens showed telomerase activity, but no hTR expression by ISH and vice versa. Other studies in different tumor types also have shown upregulation of hTR before upregulation of telomerase activity.[24,35-38] In most of these studies, hTR expression was determined by Northern blot analysis or rt-PCR. The opposite possibility, no hTR expression, but detectable telomerase activity may be due to low hTR expression, probably below the detection limit of the applied in situ technique. This hypothesis is confirmed by the fact that in the current study simultaneous analysis of hTR by rt-PCR and ISH showed that low levels of hTR were detectable in virtually all lesions, including normal cervices when rt-PCR

Table 2. Telomerase Activity in Relation to Prevalence of Telomerase Subunits

		Telomerase Activity	
		− (%)	+ (%)
ISH			
hTR	−	24 (83%)*	5 (17%)*
	+	21 (47%)*	24 (53%)*
rt-PCR			
hTR	−	1 (33%)	2 (67%)
	+	38 (62%)	23 (38%)
hTERT mRNA	+	24 (75%)†	8 (25%)†
	+	15 (47%)†	17 (53%)†

Note. r and P-values were presented if $P < .05$: Relation between positive telomerase activity and positive telomerase components.

Abbreviations: ISH, in situ hybridization; hTR, human telomeasc RNA component; rt-PCR reverse transcription polymerase chain reaction; hTERT, human telomerase reverse transcriptase.

*$r = 0.36$; $P < .01$.

†$r = 0.29$; $P < .05$.

HUMAN PATHOLOGY Volume 31, No. 10 (October 2000)

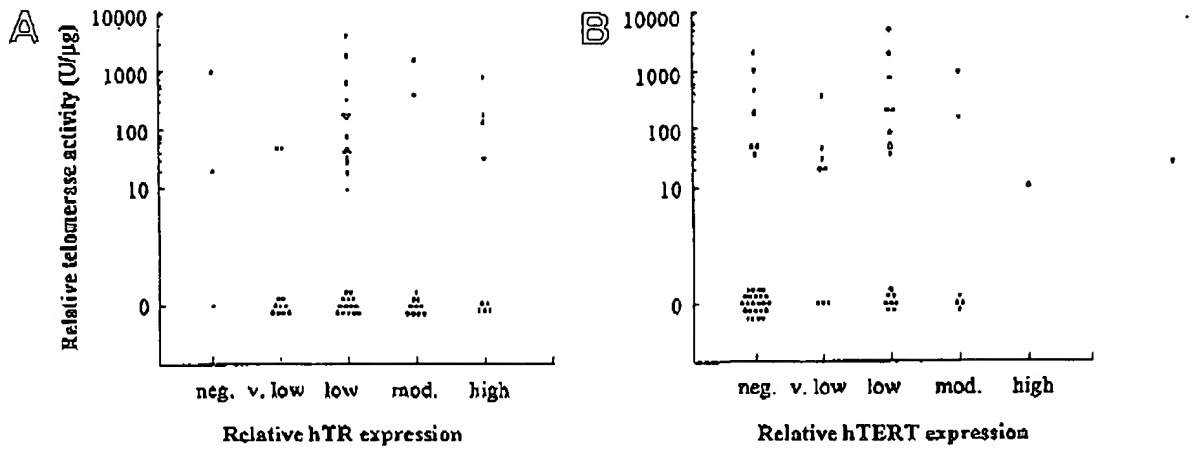

FIGURE 5. Relative hTR expression (A) and hTERT mRNA expression (B) as determined by rt-PCR in relation to telomerase activity levels as determined in the same lesions.

was used. Another explanation for no hTR but detectable telomerase activity might be heterogeneity of hTR or telomerase activity in the specimens. However, we did not observe important heterogeneity in hTR expression when using ISH. With regard to telomerase activity at present no assays, applicable on tissue sections are available to determine possible intralesional heterogeneity.

Our data on hTERT mRNA expression in cervical lesions show a relationship to positive telomerase activity, although some specimens show hTERT mRNA but no telomerase activity, and vice versa. Other studies on hTERT mRNA expression in cervical specimens, studied with rt-PCR,[38-40] are in agreement with our results. They showed hTERT mRNA expression in 0 to 100% normal cervices, whereas 80% to 100% of the cervical cancer specimens were positive for hTERT mRNA.[38-40] hTERT mRNA expression is related to presence of telomerase activity, is also related to grade of severity of the cervical lesion, and hTERT mRNA is earlier upregulated than telomerase activity.[38,39] Detectable hTERT mRNA expression, but no telomerase activity, can be attributable to upregulation of hTERT mRNA before telomerase activity. Furthermore, hTERT mRNA can consist of various splicing variants, in which deletions or inserts are found, resulting in different levels of telomerase activity.[5,41] In our study, only levels of total hTERT mRNA were determined. The opposite possibility, no hTERT mRNA expression, but detectable telomerase activity, may be attributable to low hTERT mRNA expression. From several reports, it is suggested that hTERT is the rate-limiting factor for telomerase activity,[7-10] whereas recently, other groups suggested that hTR is also necessary for functional telomerase.[11,12]

Table 3. hTR Expression in Relation to HPV Type in Normal Cervical Specimens, CIN I-III, and Cervical Cancer (CC) Specimens

Stage	Relative hTR Expression	HPV Type					
		0 (%)*	6 (%)	18, etc (%)	16 (%)	NE	Total
Normal	Negative	1 (50)			1 (50)	6	8
CIN I	Negative			4 (36)	7 (64)	2	13
	Moderate				1 (100)		1
CIN II	Negative	4 (36)		2 (18)	5 (45)	2	13
	Weak	2 (22)	1 (11)	2 (22)	4 (44)	1	10
	Moderate				4 (100)		4
	High			1 (100)			1
CIN III	Negative	1 (13)		1 (13)	6 (75)	1	9
	Weak				5 (100)		5
	Moderate				12 (100)		12
	High			2 (50)	2 (50)		4
CC	Negative			1 (100)		1	2
	Weak				3 (100)		3
	Moderate			3 (30)	7 (70)		10
	High				3 (100)		3
Total		8 (9)	1 (1)	16 (19)	60 (71)	13	98

Abbreviation: NE, not evaluable.

*Percentages are calculated per total number of evaluable patients in each class per category of hTR expression.

We showed, in our group of cervical specimens, that not only was hTERT mRNA important for telomerase activity but also elevated levels of hTR.

HPV infection is considered to be an important causative factor in cervical cancer and may also influence telomerase expression. Ex vivo studies showed that transfection of normal epithelial cervical keratinocytes with the HPV 16 E6 gene resulted in telomerase activation even before the occurrence of "crisis."[15] We and others reported on telomerase activity in cervical cancer in relation to HPV type.[17,21,22,25,42] From these studies, it appeared that telomerase activation can occur in (pre)neoplastic lesions independent of HPV. In the current study, we report for the first time hTR and hTERT mRNA expression in relation to HPV type. In contrast to other reports on telomerase activity, our data show that hTR expression, as determined by ISH, and hTERT mRNA expression, using rt-PCR, are related to the presence of oncogenic HPV type. These data suggest that hTR as well as hTERT mRNA are upregulated after infection with an ongenic HPV type, which are important for immortalization of the cell. This confirms that positive cervical specimens for ongenic HPV are more prone to develop cervical cancer.

In our previous study on telomerase activity in (pre)neoplastic lesions, we speculated that CIN lesions with increased telomerase activity may represent more "advanced stage" CIN lesions, and we also suggested that determination of telomerase activity might have value as a biomarker for progressive or persistent CIN lesions.[21] In the current study, we observed moderate or high hTR expression in 15% CIN II and 45% CIN III lesions and hTERT mRNA expression in 36% CIN II and 61% CIN III lesions. Only longitudinal nonintervention studies in CIN patients will answer the question of whether determination of telomerase activity, hTR levels using either ISH or rt-PCR, or hTERT mRNA expression indeed may have value in prediction of progression or persistence of CIN lesions.

Another relevant outcome of our previous study was that determination of telomerase activity in cervical scrapings had a low sensitivity for the detection of CIN and cervical cancer.[21] Among other factors, rapid degradation of telomerase in cervical mucus may explain this low sensitivity. In urine samples from bladder cancer patients, Müller et al[37] reported rapid degradation of telomerase activity, whereas hTR levels as determined by rt-PCR remained stable, leading to much higher sensitivity for detection of bladder cancer. Data from our study suggest that hTR levels using ISH are lower in normal cervix and CIN I in comparison to, especially, CIN III and cervical cancer. Furthermore, we and others suggest that hTERT mRNA is more often upregulated in CIN III and cervical cancer than in CIN I and CIN II. Therefore, we are evaluating the possible value of hTR or hTERT mRNA determination, either by ISH or by rt-PCR in cervical scrapings for the detection of CIN and cervical cancer.

In conclusion, our study shows frequent upregulation of hTR and hTERT mRNA expression in cervical carcinogenesis, whereas hTR and hTERT mRNA expression also appears to be upregulated earlier in cervical preneoplastic lesions when compared with telomerase activity. Additional analysis of hTR using rt-PCR showed that hTR could be detected in almost every CIN lesion and cervical cancer specimen. Our data illustrate that not only hTERT mRNA, but also upregulated hTR levels, may be important for functional telomerase. Whether or not determination of hTR levels by using ISH or determination of hTERT mRNA expression in CIN lesions may have value as possible biomarkers for persistent/progressive disease has to be evaluated in larger longitudinal studies without therapeutic interventions.

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